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(54) Title: ASSAY FOR THE DETECTION OF ANTIBODIES AGAINST p53

(57) Abstract

A method is provided for detecting antibodies capable of binding to p53 protein using surface plasmon resonance biosensor having immobilised thereon p53 peptides.

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ASSAY FOR THE DETECTION OF ANTIBODIES AGAINST p53

This patent application claims the benefit under Title 35, United States Code, §119(e) of U.S. Provisional Patent Application No. 60/028,533 filed October 7, 1996.

FIELD OF INVENTION

The present invention relates to a method for the detection of antibodies capable of binding to p53 protein, particularly human p53 protein.

BACKGROUND

Wild-type p53 gene is a tumor suppressor gene encoding cellular wild-type p53 protein. Endogenous wild-type p53 protein has been found to be lacking (either absent or mutated) in a variety of cancer cells, such as breast carcinoma cells and lung carcinoma cells, and it has been found that administration of wild-type p53 gene into cancer cells lacking endogenous wild-type p53 protein can be used to treat cancer by suppression of the neoplastic phenotype. See U.S. Patent 5,532,220.

In carrying out p53 gene therapy, it would be highly desirable to monitor serum samples from patients prior and subsequent to exposure to the exogenous p53 gene to determine if an immunogenic response has been mounted in response to this exposure. This could be accomplished by use of an assay to screen serum samples for antibodies capable of binding to p53. In addition, since certain cancer patients produce antibodies against the mutant p53 that they are producing, it would also be desirable to have a diagnostic assay to test serum samples for the presence of antibodies capable of binding to p53.

In PCT patent publication WO 94/10306 Soussi *et al.* disclosed an assay for using biotinylated peptides in an enzyme linked immunosorbent assay ("ELISA") format to detect p53 antibodies in patient serum samples. However, it would be highly desirable to provide a new, improved assay for detecting p53

antibodies. The major areas where improvement is needed are as follows. First, there is a critical need for an assay that is better suited for detecting lower affinity antibodies. Methods employing ELISAs have difficulty in this regard because ELISAs require multiple incubation steps followed by wash cycles during which lower affinity antibodies can wash away. Second, there is a need for an assay which is less labor intensive than the methods utilizing an ELISA format. Third, there is a need to significantly reduce analysis time, preferably to less than 10 minutes/sample, to allow for high throughput analyses. As mentioned above, ELISAs require multiple incubation steps followed by wash cycles, and therefore the materials used in the assay cannot be regenerated for analysis of subsequent samples.

SUMMARY OF THE INVENTION

The present invention meets the foregoing needs by providing a method for detecting antibodies capable of binding to p53 protein comprising:

A) immobilizing a peptide directly onto a flowcell of a sensorchip in a biosensor, said peptide capable of specifically being bound to an anti-p53 antibody,

B) obtaining a serum sample from a patient to be tested and diluting the serum sample in a suitable buffer,

C) contacting the diluted serum sample with the immobilized peptide, and

D) measuring binding of antibodies to the immobilized peptide by means of the biosensor.

In the method of the present invention, it is preferred that a plurality of selected immunogenic peptides are used, each immobilized directly on its own separate flowcell. Preferably, a first peptide is selected from the amino terminal region of the p53 protein and a second peptide is selected from the carboxy terminal region of the p53 protein. Preferably still, the present invention employs one or more of the following four peptides (or peptides having substantial sequence identity thereto):

a) a peptide comprising SEQ ID NO: 1 (C11-35, corresponding to residues 11-35 of the human p53 protein: H-CEPPLSQETFSDLWKLLPENNVLSPL-Amide), or a peptide having substantial sequence identity thereto;

b) a peptide comprising SEQ ID NO: 2 (C40-65, corresponding to residues 40-65 of the human p53 protein: H-CMDDLMLSPDDIEQWFTEDPGPDEAPR-Amide), or a peptide having substantial sequence identity thereto;

c) a peptide comprising SEQ ID NO: 3 (C346-370, corresponding to residues 346-370 of the human p53: protein H-EALELKDAQAGKEPGGSRAHSSHLK-Amide), or a peptide having substantial sequence identity thereto; and

d) a peptide comprising SEQ ID NO: 4 (C371-390, corresponding to residues 371-390 of the human p53: protein H-CSKKGQSTSRHKKLMFKTEGP-Amide), or a peptide having substantial sequence identity thereto.

(The above sequences are listed using standard one-letter amino acid symbols; *see e.g.*, Lehninger, *Principles of Biochemistry*, Worth Publishers, Inc. 6th Ed. 1988, p. 96).

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a graphical depiction of response units vs. dilution factor to show the relative sensitivity of the four noted peptides to antibodies in normal human serum.

Figure 2 is a graphical depiction of response units vs. dilution factor to show the relative sensitivity of the four noted peptides to antibodies in normal pig serum.

Figures 3A and 3B are graphical depictions showing antibody binding (to the four noted peptides) for normal human serum samples. In the Figures, the abbreviation NHS = pooled normal human serum, and Pos = sheep polyclonal Ab 1:200 into NHS. For peptide C11-35 the mean is 41.1 and the standard deviation (SD) is 29.8; for peptide C40-65 the mean is 30.6 and SD is 27.0; for peptide C346-370 the mean is 38.9 and SD is 30.9; and for peptide C371-390 mean is 38.0 and SD is 18.7.

Figures 4A and 4B are graphical depictions showing antibody binding (to the four noted peptides) for serum samples from normal pigs. In the Figures, NPS

= pooled normal pig serum 1:40 dilution, and Pos = sheep polyclonal Ab 1:200 into NPS. For peptide C11-35 the mean is 50.9 and SD is 17.7; for peptide C40-65 the mean is 52.9 and SD is 15.6; for peptide C346-370 the mean is 35.0 and SD is 16.6; and for peptide C371-390 mean is 38.8 and SD is 14.8.

DETAILED DESCRIPTION OF THE INVENTION

All references cited herein are hereby incorporated in their entirety by reference.

In the method of the present invention selected immunogenic peptides are immobilized *directly* onto flowcells of a sensorchip in a biosensor. Previous assays such as the assay of Soussi *et al* employed an ELISA format utilizing immobilized streptavidin to capture biotinylated peptides, and therefore the peptide immobilization was indirect. In the preferred embodiment described in the example herein (described in more detail below), the direct immobilization technique comprises the use of amine and/or thiol chemistry to directly immobilize N-labelled cysteine peptides.

The present inventors have found that their method of direct immobilization has several critical advantages, including far better detection of lower affinity antibodies (resulting in significantly more precise analyses). In addition, the present invention significantly reduces analysis time, allowing for high throughput analyses. Unlike previous assays, the assay of the present invention with its direct immobilization feature permits high throughput by virtue of the fact that, after a given sample is analyzed, the materials used in the assay can quickly be regenerated for analysis of subsequent samples. The direct immobilization techniques of the present invention permit fast and easy washing with reagents such as a HCl-SDS solution (preferably 25 mM HCl-0.25% SDS).

The present invention also provides an assay that requires only a small amount of serum, *e.g.*, less than 5 µl in order to carry out an analysis. Previous assays using a mircotiter plate ELISA format can require much more serum from the patient, *e.g.*, when there is a need to characterize the antibodies produced by the patient. In this regard, the present inventors have also found that their new method allows for the isotypic identification of the antibodies that are detected

without requiring additional samples. (The assay of Soussi *et al* requires multiple applications of samples to obtain isotype determination).

Preferred peptides for use in the method of the present invention are selected from the group consisting of:

a) Peptide C11-35, corresponding to residues 11-35 of the human p53 protein: H-CEPPLSQETFSDLWKLLPENNVLSPL-Amide;

b) Peptide C40-65, corresponding to residues 40-65 of the human p53 protein: H-CMDDLMLSPDDIEQWFTEDPGPDEAPR-Amide;

c) Peptide C346-370, corresponding to residues 346-370 of the human p53: protein H-CEALELKDAQAGKEPGGSRAHSSHLK-Amide; and

d) Peptide C371-390, corresponding to residues 371-390 of the human p53: protein H-CSKKGQSTSRHKKLMFKTEGP-Amide.

Peptides having substantial sequence identity to the above-mentioned peptides can also be employed in preferred embodiments. Here, "substantial sequence identity" means that two peptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap weights, share at least 80 percent sequence identity, preferably at least 90 percent sequence identity, more preferably at least 95 percent sequence identity or more. Preferably, residue positions which are not identical differ by conservative amino acid substitutions. For example, the substitution of amino acids having similar chemical properties such as charge or polarity are not likely to effect the properties of a protein. Examples include glutamine for asparagine or glutamic acid for aspartic acid.

In preferred embodiments of the present invention a plurality of selected immunogenic peptides can be directly immobilized, each on its own separate flowcell. For instance, a first peptide can be selected from the amino terminal region of the p53 protein and a second peptide can be selected from the carboxy terminal region of the p53 protein. In a more specific example, the first peptide comprises SEQ ID NO: 1 (H-CEPPLSQETFSDLWKLLPENNVLSPL-Amide), and the second peptide comprises SEQ ID NO: 4 (H-CSKKGQSTSRHKKLMFKTEGP-Amide). In addition, a third peptide comprising SEQ ID NO: 2 (H-

CMDDLMLSPDDIEQWFTEDPGPDEAPR-Amide) and a fourth peptide comprising SEQ ID NO: 3 (H-CEALELKDAQAGKEPGGSRAHSSHLK-Amide) can be used.

Phosphorylated peptides can also be used in the method of the present invention. Specifically, a serine residue can be replaced by a phosphoserine residue, and a threonine residue can be replaced by a phosphothreonine residue. By way of example, peptides that have been used successfully in the method of the present invention have been selected from the group consisting of:

a) a peptide comprising SEQ ID NO: 1, or a peptide having substantial sequence identity thereto, wherein all serine residues in the peptide are replaced by phosphoserine residues; and

b) a peptide comprising SEQ ID NO: 3, or a peptide having substantial sequence identity thereto, wherein all serine residues in the peptide are replaced by phosphoserine residues.

The biosensor used in the present invention is preferably a BIAcore 2000™ from BIAcore (Uppsalla, Sweden) (previously available from Pharmacia). The BIAcore 2000™ operates on a principle of surface plasmon resonance and allows for high throughput analyses. (*See, e.g.*, Hodgson, Bio/Technology vol. 12, Jan. 1994 for a review of biosensors). Other biosensors, *e.g.*, a BIAcore X from BIAcore or a IAsys biosensor from Fisons, could be used in connection with the present invention. In analyzing the results of the assay, it is preferred that the amount of antibody that binds to each peptide is directly proportional to the biosensor signal (*e.g.*, the response units that are reported by the BIAcore 2000™ biosensor).

In carrying out the method of the present invention, it is preferred that the serum samples to be analyzed are diluted within a range of 1:2 through 1:500 (more preferably within a range of 1:2 through 1:500, more preferably still at a dilution of 1:40) in a HEPES buffered saline solution containing the detergent P-20 and carboxymethylated dextran, prior to the step of simultaneously contacting the serum samples with the immobilized peptides.

The broad scope of this invention can be understood with reference to the following example, which is not intended to limit the invention to specific embodiments.

EXAMPLE

In the specific example illustrated herein, each peptide is directly immobilized onto the surface of separate flowcells of a sensorchip in the BIAcore 2000™ (BIAcore, Uppsalla, Sweden). Serum samples are diluted (1:40 in a HEPES buffered saline solution containing the detergent P-20 and carboxymethylated dextran in this example) and allowed to bind to each immobilized peptide simultaneously. The amount of antibody that binds to each peptide is directly proportional to the response units that are reported by the instrument. The flowcells are regenerated simultaneously using 25 mM HCl-0.25% SDS to remove the bound antibody and the next sample is then analyzed.

The data presented below demonstrate that in the assay of the present invention p53 antibodies can be precisely detected in human and pig serum samples, and further that the assay can be performed in an automated format that allows a high throughput of sample analysis. One sensorchip can be used to analyze pre-dose and post-dose samples from multiple individuals along with a positive control (*e.g.*, sheep polyclonal anti-p53 antibody from Oncogene Sciences or the equivalent) and a negative control (pooled serum) analyzed at the beginning and at the end of each assay.

Initial criteria for samples to be considered "POSITIVE" for the presence of anti-human p53 antibodies are: 1) binding above threshold to any of the immobilized peptides and 2) that the binding is verified to be due to immunoglobulins. Alternatively, isotyping reagents anti-human IgG1, anti-human IgG2, anti-human IgG3, anti-human IgG4 anti-human IgA, and anti-human IgM (from ICN or equivalent) could be added in sequence and the binding monitored. An individual is considered to have developed antibodies against p53 if there is binding above threshold and at least a 2-fold increase in response units when comparing a sample obtained after administration of a delivery vehicle (*e.g.*, vector) containing the p53 gene with a sample obtained from the same individual prior to administration. (With regard to delivery vehicles, construction of an adenovirus p53 gene therapy vector has been described by Wills *et al. Human Gene Therapy 5* : 1079 (1994)).

II. Selection of Synthetic Peptides

The following synthetic peptides (each synthesized with an N-terminal cysteine residue to promote coupling) were selected for the assay used in the present example.

a) Peptide C11-35, corresponding to residues 11-35 of the human p53 protein: H-CEPPLSQETFSDLWKLLPENNVLSPL-Amide (SEQ ID NO: 1).

b) Peptide C40-65, corresponding to residues 40-65 of the human p53 protein: H-CMDDLMLSPDDIEQWFTEDPGPDEAPR-Amide (SEQ ID NO: 2);

c) Peptide C346-370, corresponding to residues 346-370 of the human p53: protein H-CEALELKDAQAGKEPGGSRAHSSHLK-Amide (SEQ ID NO: 3);

d) Peptide C371-390, corresponding to residues 371-390 of the human p53: protein H-CSKKGQSTSRHKKLMFKTEGP-Amide (SEQ ID NO: 4).

III. Immobilization of Synthetic Peptides and Regeneration Stability

Peptide C371-390 was immobilized using amine coupling. For this immobilization, the peptide was diluted to a concentration of 100 µg/ml into 20 mM borate buffer at pH 8. The remaining three peptides were immobilized using thiol coupling. Peptide C11-35 was diluted to a concentration of 700 µg/ml into 10 mM sodium acetate buffer pH 4; peptide C40-65 was diluted to a concentration of 1 mg/ml into 10 mM sodium acetate buffer at pH 4.0; and peptide C346-370 was diluted to a concentration of 500 µg/ml into 10 mM MES buffer at pH 5.

A summary of the peptide immobilizations is shown in Table 1. (Tables 1 through 7 are grouped together after section X below). The data in Table 1 indicate that there is variability in the peptide immobilizations. This variability does not effect the precision of the assay (discussed in section V below). These data suggest that it is important to verify that the amount of peptide immobilized is sufficient to allow binding of the positive control antibody at a 1:200 dilution. The data on stability during regeneration with 25 mM HCl and 0.25% SDS (Table 1)

indicate that the immobilized peptide surfaces are stable through at least 147 regeneration cycles since the binding among replicates of the positive control assayed after multiple regeneration cycles was within 20.4 % coefficient of variability ("CV") for all peptides.

IV. Linearity/Sensitivity

The linearity of this assay was determined by assaying the sheep positive control antibody at various dilutions and plotting the response units ("RU") versus the dilution factor of the sample. (In the BIAcore 2000™ biosensor, 1000 response units (or resonance units) is equivalent to 1 ng/mm^2; *see, e.g.*, BIAcore Methods Manual). As shown in Figure 1, the response of the positive control diluted in pooled normal human serum is dependent on the concentration of the antibody from a 1:10 initial dilution through a 1:2560 dilution. The C346-370 peptide is not recognized as strongly by the positive control as are the remaining three peptides. These data indicate that the assay is sensitive over approximately a 2-log concentration range. The data in Figure 2 demonstrate that the sensitivity is similar when the sheep positive control is diluted into pooled normal pig serum. In both sera, the positive control reacts most strongly with the C11-35 peptide, followed in order of reactivity by the C40-65 peptide, the C371-390 peptide, and finally with the less well recognized C346-370 peptide.

It should be noted here that lower affinity antibodies typically have more rapid dissociation rates than higher affinity antibodies, and these rates can be monitored on a biosensor in accordance with the present invention. (In contrast, an ELISA is only able to detect the amount of antibody present at the final detection phase and is unable to monitor dissociation as it occurs).

V. Precision

The precision of this assay for human and pig serum samples was determined by adding the sheep anti-p53 positive control antibody (Oncogene Sciences) at three dilutions (1:50, 1:100, and 1:200) into 2.5% pooled normal human serum and pooled normal pig serum (diluted into HEPES buffered saline containing the detergent P-20 and soluble carboxymethylated dextran) and then assaying multiple aliquots during the same assay (intra-assay precision) and for multiple assays (inter-assay precision). At least 4 aliquots of each dilution were analyzed per day for 5 days. The results for intra-assay precision in human

serum samples can be found in Table 2, and the results for inter-assay precision are in Table 3. The intra-assay and inter-assay precision results from pig serum samples can be found in Tables 4 and 5 respectively.

The results from the samples analyzed for intra-assay precision in human serum all ranged within 2% CV for each dilution of positive control binding to each peptide. The samples analyzed for inter-assay precision in human serum had CVs that ranged within 16.3% for all dilutions for the three peptides that are fully recognized by the positive control. The CVs from peptide C346-370 ranged from 22.6% to 37.7% and therefore the assay is less precise for this peptide due to the low binding to that peptide by this polyclonal sheep antibody.

The samples analyzed for intra-assay precision in pig serum (Table 4) were all analyzed with CVs in the range of 2.6% to 16.1%. The CVs from the samples analyzed for inter-assay precision were all within the range of 3.6% to 17.0%.

Taken together, these results indicate that the assay is precise for both pig and human serum samples. Further, the variability associated with the peptide immobilization does not significantly affect the inter-assay precision of this assay, since these results were obtained using the multiple immobilizations described in Table 1.

VI. Determination of Threshold

To determine the threshold for binding to the synthetic peptides (for a diluted sample to be considered positive for the presence of antibodies capable of binding p53), serum samples from normal human volunteers and normal pigs were analyzed. For instance, the samples in the example above were diluted 1:40 into HEPES buffered saline containing carboxymethylated dextran and the detergent P-20 to reduce non-specific binding. The binding of normal human serum samples is shown in Figures 3A and 3B. The threshold of a positive sample binding to each peptide is as follows: C11-35 = 131.5; C40-65 = 130.4; C346-370 = 111.6; and C371-390 = 94.1. The threshold is defined as the mean + 3x standard deviation from the binding of the normal human serum samples (at a specific serum sample dilution). Any sample with binding greater than the threshold for any of the immobilized peptides will be considered reactive for the presence of antibodies against human p53.

The binding of serum samples from normal pigs is shown in Figures 4A and 4B. The threshold for each peptide is as follows: C11-35 = 104.1; C40-65 = 99.6; C346-370 = 84.9; and C371-390 = 83.1. The threshold is again defined as mean + 3x the standard deviation from the binding observed with the normal pig samples.

Samples will be considered positive for the presence of antibodies capable of binding to human p53 if the following conditions are met: 1) there is binding greater than threshold to any of the immobilized peptides and 2) the binding is found to be a result of immunoglobulin (verified by an increase in binding when an anti-species immunoglobulin is added to the captured anti-p53 antibody).

In addition, individuals will be considered to have generated antibodies in response to treatment with a p53-containing vector if the following conditions occur: 1) there is binding greater than threshold to any of the immobilized peptides, 2) the binding is found to be a result of immunoglobulin (verified by an increase in binding when an anti-species immunoglobulin is added to the captured serum component), and 3) there is at least a 2-fold increase in binding observed from a sample obtained after dosing with the vector compared with a sample obtained prior to dosing.

(In summary, the human or pig serum samples will be considered to be positive if there is binding to any of the 4 synthetic peptides greater than threshold and that binding is shown to be a result of immunoglobulin by increased binding with an anti-species specific immunoglobulin).

VII. Specificity

The specificity of this assay was determined by adding a panel of antibodies and cytokines to 2.5% pooled pig serum and monitoring the binding to each of the 4 immobilized peptides. None of the test compounds except the sheep anti-p53 positive control antibody demonstrated any binding to any of the immobilized peptides. These results indicate that this assay is specific for antibodies against human p53.

VIII. Effect of Freeze/Thaw

To test whether samples can be reassayed following multiple freeze/thaw cycles, aliquots of dilutions of the sheep positive control antibody were prepared in 100% pooled normal human serum and subjected to from 1 to 5 freeze/thaw cycles prior to analysis for the presence of anti-p53 antibodies. The results of these analyses shown in Table 6 indicate that antibody samples are stable through at least 5 freeze/thaw cycles. These data indicate that samples can be re-analyzed up to at least 4 times without effecting the result that is obtained.

IX. Reactivity of Serum Obtained from Selected Cancer Patients

Serum samples from cancer patients were tested. As shown in Table 7, two of the 7 samples were verified to contain antibodies capable of binding to human p53. The verification was performed by re-assaying initially positive samples and testing whether the binding to a synthetic peptide could be repeated and also whether there was additional binding upon addition of anti-human IgG/IgA/IgM (Kirkegaard and Perry, Gaithersburg, MD) (or equivalent).

X. Summary

The data contained herein indicate that the assay of the present invention is precise, sensitive, specific, and unaffected by multiple freeze/thaw cycles.

Table 1 Peptide Immobilization Reproducibility and Stability

	Amount of Peptide Immobilized in RU			
Date of Immobilization	C11-35	C40-65	C346-370	C371-390
DAY 1	1175	871	1006	1051
DAY 14	515	415	682	1607
DAY 18	833	528	866	1072
DAY 19	514	286	838	1593
DAY 22	529	638	655	1493
MEAN	713.2	547.6	809.4	1363.2
SD	291.8	223.1	143.8	279.0
%CV	40.9	40.7	17.8	20.5

B. Peptide Stability After Regeneration

Regneration Cycle #	C11-35	C40-65	C346-370	C371-390
2	3715.8	1513.2	68.6	712.2
53	4866.5	1671.1	77.9	838.1
147	4147.7	2189.9	51.4	745.6
MEAN	4243.3	1791.4	66.0	765.3
St. Dev.	581.3	354.0	13.4	65.2
%CV	13.7	19.8	20.4	8.5

Stability was determined by assaying aliquots of the sheep anti-p53 antibody at 1:50 into 2.5% normal pooled human serum after multiple regeneration cycles using 25 mM HCl and O. 25% SDS

Table 2 Intra-assay Precision in Normal Human Serum

	Binding in RU to Each Peptide			
	C11-35	C40-65	C346-370	C371-390
Pos Con 1:50	5131.4	1819.3	126.4	1114.7
	5049.6	1836.2	124.5	1126.8
	5152.1	1870.3	125.3	1152.2
	5054.7	1860.2	124.9	1148.3
Mean	5097.0	1846.5	125.3	1135.5
StDev	52.5	23.1	0.8	17.8
%CV	1.0	1.3	0.7	1.6
Pos Con 1:100	2627.0	980.8	66.2	553.2
	2633.0	978.5	66.1	552.6
	2555.1	1004.4	65.3	563.4
	2610.0	971.3	66.0	553.8
Mean	2606.3	983.8	65.9	555.8
StDev	35.5	14.3	0.4	5.1
%CV	1.4	1.5	0.6	0.9
Pos Con 1:200	1295.4	488.9	36	275.1
	1292.4	486.4	36	274.4
	1305.9	483.4	35.6	274.9
	1268.8	504.7	35.4	280.9
Mean	1290.6	490.9	35.8	276.3
StDev	15.7	9.5	0.3	3.1
%CV	1.2	1.9	0.8	1.1

Table 3 Inter-assay Precision in Normal Human Serum

		Binding in RU to Each Peptide			
		C11-35	C40-65	C346-370	C371-390
Pos Con 1:50	Day 1	4345.9	1708.9	71.2	782.4
	Day 2	4674.3	1856.7	72.6	805.4
	Day 3	3204.0	1928.7	AA	989.0
	Day 4	4284.8	2027.5	89.7	1001.5
	Day 5	5097.0	1846.5	125.3	1135.5
Mean		4321.2	1873.7	89.7	942.8
StDev		702.9	117.1	25.2	147.8
%CV		16.3	6.3	28.1	15.7
Pos Con 1:100	Day 1	2518.7	991.1	44.0	452.1
	Day 2	2251.9	918.3	43.6	441.0
	Day 3	1907.5	842.6	AA	479.8
	Day 4	2148.9	854.5	43.4	480.7
	Day 5	2606.3	983.8	65.9	555.8
Mean		2286.7	918.1	49.2	481.9
StDev		282.8	69.6	11.1	44.8
%CV		12.4	7.6	22.6	9.3
Pos Con 1:200	Day 1	1285.6	588.1	27.1	257.0
	Day 2	1272.9	587.3	23.6	243.5
	Day 3	975.3	511.1	AA	240.2
	Day 4	941.5	496.2	13.1	232.9
	Day 5	1290.6	490.9	35.8	276.3
Mean		1153.2	534.7	24.9	250.0
StDev		178.3	48.9	9.4	17.1
%CV		15.5	9.2	37.7	6.8

AA = Analytical error, data not used

Listed means represent mean from each of 5 days with 4 replicates/day

Table 4 Intra-assay Precision in Normal Pig Serum

	C11-35	C40-65	C346-370	C371-390
Pos Con 1:50	3948.9	2342.9	140.1	1435.1
	3603.7	2323.8	135.5	1395.1
	3639.9	2472.0	129.1	1395.3
	3693.5	2377.7	128.6	1360.9
	3277.1	2096.1	121.5	1264.6
	2574.5	1741.4	113.0	1148.1
Mean	3456.3	2225.7	128.0	1333.2
StDev	482.5	267.8	9.7	107.5
%CV	14.0	12.0	7.6	8.1
Pos Con 1:100	1901.0	1168.1	77.3	691.5
	1722.6	1079.6	74.6	662.2
	1921.7	1175.2	71.6	673.7
	1755.3	1154.9	69.5	665.5
	1948.9	1094.4	69.1	653.3
	1949.0	1073.0	68.2	642.1
Mean	1866.4	1124.2	71.7	664.7
StDev	100.9	46.8	3.6	17.0
%CV	5.4	4.2	5.0	2.6
Pos Con 1:200	922.9	579.3	48.1	349.3
	958.0	552.8	46.9	337.4
	872.8	572.4	42.7	335.2
	916.1	544.4	42.7	330.3
	742.7	412.4	39.2	287.2
	615.6	397.5	35.9	270.9
Mean	838.0	509.8	42.6	318.4
StDev	132.3	82.3	4.6	31.5
%CV	15.8	16.1	10.8	9.9

Table 5 Inter-assay Precision in Normal Pig Serum

		C11-35	C40-65	C346-370	C371-390
Pos Con 1:50	Day 1	3456.3	2225.7	128.0	1333.2
	Day 2	4724.4	2809.9	144.2	1369.6
	Day 3	4675.3	1766.7	134.2	1093.5
	Day 4	4167.3	2473.8	132.3	1282.4
	Day 5	4090.3	2517.8	136.1	1351.4
Mean		4222.7	2358.8	134.9	1286.0
StDev		515.9	390.6	6.0	112.4
%CV		12.2	16.6	4.4	8.7
Pos Con 1:100	Day 1	1866.4	1124.2	71.7	664.7
	Day 2	2284.7	1301.6	77.7	706.9
	Day 3	2115.9	916.5	71.0	530.1
	Day 4	2117.5	1175.3	73.2	612.9
	Day 5	2075.6	1212.9	74.7	685.8
Mean		2092.0	1146.1	73.7	640.1
StDev		149.6	143.8	2.7	70.7
%CV		7.2	12.5	3.6	11.0
Pos Con 1:200	Day 1	838.0	509.8	42.6	318.4
	Day 2	1119.5	619.6	43.7	344.0
	Day 3	1078.1	476.9	42.6	281.5
	Day 4	1083.7	619.8	60.7	306.5
	Day 5	1118.1	573.5	43.3	296.6
Mean		1047.5	559.9	46.6	309.4
StDev		118.6	64.7	7.9	23.6
%CV		11.3	11.6	17.0	7.6

Table 6 Effect of Freeze/Thaw Cycles on Sample Stability

	Binding in RU to Identified Peptide			
Freeze/Thaw Cycle #	C11-35	C40-65	C346-370	C371-390
Cycle 1	808.0	236.3	10.0	850.9
Cycle 2	898.3	251.4	11.9	930.4
Cycle 3	1016.7	281.7	13.1	1063.8
Cycle 4	915.1	254.2	11.2	943.1
Cycle 5	910.9	239.8	10.6	911.5
%CV	8.1	7.1	10.8	8.3
%Change	12.7	1.5	6.0	7.1
Mean	909.8	252.7	11.3	939.9
ST Dev	74.1	17.9	1.2	77.7
% CV	8.1	7.1	10.8	8.3

Table 7 Screening of Serum Samples from Cancer Patients

Patient ID	Binding to Immobilized Peptides in RU C11-35	C40-65	C346-370	C371-390	Binding of anti-hu IgG/IgM/IgA in RU C11-35	C40-65	C346-370	C371-390
1	0	31.9	0	13.9				
2	771.0	203.6	16.4	442.2				
2 (repeat)	814.0	157.3	10.8	438.2	222.3	56.4	14.0	131.7
3	56.1	83.4	27.4	135.2				
3 (repeat)	0	70.3	11.3	92.7	37.7	12.4	8.9	0
4	203.5	218.9	3.3	0				
4 (repeat)	192.0	180.3	0	11.9	74.1	74.8	0	2.6
5	34.8	155.3	1.1	59.6				
5 (repeat)	37.9	133.4	0	70.0	73.7	15.4	10.1	22.7
6	113.8	118.9	49.4	62.2				
7	32.3	22.1	16.5	7.2				

Patient ID	Result	
1	NEGATIVE	(no initial reactivity above threshold to any peptide)
2	POSITIVE	(reactivity against 3/4 peptides, confirmed as Ab)
3	NEGATIVE	initial reactivity against peptide 4 could not be confirmed as Ab)
4	POSITIVE	reactivity against 2/4 peptides, confirmed as Ab)
5	NEGATIVE	initial reactivity against peptide 2 could note confirmed as Ab)
6	NEGATIVE	(no initial reactivity above threshold to any peptide)
7	NEGATIVE	(no initial reactivity above threshold to any peptide)

Note: Samples considered positive if there is binding greater than threshold to any of the peptides and binding is a result of antibody
Thresholds: C11-35 = 131.5; C40-65 = 130.4; C346-370 = 111.6; C371-390 = 94.1
Any RU <0 is reported as 0

I. Specific Procedure for Immobilization of p53 peptides according to preferred embodiment of the present invention

MATERIALS:

1. BIAcore 2000 biosensor @ 25 °C

2. Four peptides corresponding to the following amino acid sequences of human p53 protein with an additional N-terminal cysteine residue :

(a) C11-35 A.A. H-CEPPLSQETFSDLWKLLPENNVLSPL-AMIDE (MW = 2969.42)
Stock: Prepare a 1-2 mg/ml in 20 mM NaOAc pH 4 ; aliquot and store @ -20 °C.
Working sol'n: Dilute to 700 ug/ml in 20 mM NaOAc pH 4 from stock.

(b) C40-65 A.A. H-CMDDLMLSPDDIEQWFTEDPGPDEAPR-AMIDE
(MW = 3121.4)
Stock: Prepare 2 mg/ml fresh in deionized water and vortex vigorously; Dilute 1:2 with 20 mM NaOAC pH 4 from stock ;Vortex.
Working sol'n: Dilute to 1 mg/ml in 20 mM NaOAC pH 4 from fresh stock; Vortex.

(c) C346-370 A.A. H-CEALELKDAQAGKEPGGSRAHSSHLK-AMIDE
(MW = 2719.02)
Stock: Prepare a 2-3 mg/ml stock in 20 mM MES pH 6.5 and vortex; aliquot and store @ -20 °C.
Working sol'n: Dilute to 500 ug/ml in 20 mM MES pH 5 from stock;Vortex.

(d) C371-390 A.A. H-CSKKGQSTSRHKKLMFKTEGP-AMIDE (MW = 2376.8)
Stock: Prepare 1-2 mg/ml in 20 mM MES pH 6.5 and vortex; aliquot and store @ -20 °C.
Working sol'n: Dilute to 100 ug/ml in 20 mM Borate pH 8 from stock.Vortex.

3. Sensorchip CM5- (Research grade or Certified) - BIAcore

4. Amine Coupling reagents include:

Amine Coupling Kit- BIAcore (Code BR-1000-50) consisting of:

NHS: N-hydroxysuccinimide @ 11.5 mg/ml
EDC: N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide @ 75 mg/ml
1 M Ethanolamine-HCL pH 8.

5. Thiol Coupling reagents:

NHS and EDC: See Amine coupling Kit (Pharmacia Biosensor) above
100 mM Sodium Borate pH 8.5 : Fisher or equivalent
PDEA: Pharmacia Biosensor (Code BR-1000-58) Prepare 80 mM PDEA by dissolving 0.036 gms. into 2.0 ml 0.1 M Borate pH 8.5 (Note: This must be prepared immediately before use and Discarded after 2 hours after preparation) (Also note: one can use other equivalent reagents (besides PDEA for introducing reactive disulfide groups onto amino groups).
Formic Acid: Aldrich or equivalent
Sodium Formate: Aldrich or equivalent; Prepare a 100 mM formate by dissolving 0.061 gms. sodium formate into 25 ml deionized water and adjust pH with Formic acid to pH 4.3
Sodium chloride (NaCl) : Fisher or equivalent;
Cysteine: Sigma Chemical or equivalent; Prepare a 50 mM cysteine / 1 M sodium chloride by dissolving 0.061 gms. cysteine and 0.5844 gms. NaCl into 10.0 ml 0.1 M formate pH 4.3 (Note: This must be prepared immediately before use- Discard 2 hours after preparation)

6. Running buffer for BIAcore (HBS w/ P-20)
HEPES buffered saline containing P-20 surfactant(per 1 L):
2.38 g HEPES (Fisher or equivalant)
8.77 g NaCL (Fisher or equivalent
1.27 g EDTA (Sigma or equivalent)
0.5 ml P-20 (BIAcore-Cat# BR-1000-54)
pH to pH 7.4 with NaOH
Filter through 0.2 u m filter
Degas for 15 minutes
Re-pH and adjust to pH 7.4 if necessary

7. Regeneration solution (25 mM HCL / 0.25 % SDS)
   50 mM HCL prepared from a 1 M HCL stock (Anachemia or equivalent)
   0.5% SDS (BIAcore - BIAdesorb solution 1 Cat# BR-1002-22)
   Dilute 1 part 50 mM HCL with 1 part 0.5%SDS.

PROCEDURE:

1. Using the BIA 2000, immobilized C11-35 peptide on FC 1, C40-65 peptide on FC 2 and C346-370 peptide on FC 3 using Thiol-coupling chemistry and the following conditions:

- Flow rate = 10 ul / minute
- NHS/EDC = 20 ul
- PDEA = 40 ul
- Peptide = 50 ul (Except C40-65 peptide: Injected 100 ul)
- Cysteine / 1M NaCL = 40 ul
- 25 mM HCL / 0.25% SDS = 10 ul

2. Using the BIA 2000, immobilize C371-390 peptide on FC 4 using NHS/EDC amine coupling chemistry and the following conditions:

- Flow rate = 5 ul / minute
- NHS/EDC = 10 ul
- C371-390 peptide = 35 ul
- Ethanolamine = 35 ul
- 25 mM HCL / 0.25 %SDS = 10 ul

II. Procedure for detection of p53 antibodies in serum

Materials:

1. Sample Diluent : HBS w/ P-20 and CM-D;
   HEPES buffered saline containing 1 mg/ml carboxy-methyl dextran (Fluka or equivalent)
2. Regeneration solution - 25 mM HCL / 0.25 % SDS; Prepared fresh daily

3. Micro-fuge Filters, sterile ,0.2 µm (MSI - Cat# CFA02015sm or equivalent)
4. Negative Control: Serum to match matrix of samples analyzed and 0.2 µ filtered.
5. Positive Control : Sheep polyclonal anti-p53 antibody (Oncogene Sci. -Cat# AB-7) diluted 1:100 in Negative control.
6. Serum Samples: Diluted 1:40 in sample diluent and 0.2 µm filtered.

Protocol:

1. Prepare negative control by diluting serum that matches the sample dilution to be analyzed in sample diluent (*e.g.*, 1:40 dilution in the example above).
2. Prepare Positive control by diluting Sheep anti-p53 pAb 1:100 in negative control serum.
3. Dilute unknown samples in sample diluent and 0.2 µm filter (*e.g.*, 1:40 dilution in the example above).
4. Analyze samples and controls following method below using BIAcore 2000:

Flow rate : 10 µl / minute
Flow cell: 1,2,3,4
Flowpath: FC 1,2,3,4
Unknown Sample volume : 40 µl
Report point: 4.5 minutes from baseline
Regeneration sol'n: 10 µl

5. An automated method is run and data collected as response units (RU) measured for each sample and control.

Data Analysis:

The Sample response units (RU) measured for each peptide are then determined to be "Positive" by the following criteria:

1- If the sample RU is less than each peptide threshold of all four peptides for that specific sample dilution, then the sample is considered "Negative" .

2- If the sample RU is greater than or equal to the peptide Threshold RU for that peptide for that specific sample dilution, then the sample is repeated.

3- The repeat sample is confirmed by the addition of a anti-species specific antibody to verify that the RU increase measured is due to antibody.

4- If the RU of the anti-species-specific antibody is greater than 50 RU, the sample is considered "Positive" for p53 antibodies.

(Alternatively, if a sample is limited, the confirming anti-species specific antibody or isotyping reagents can be added after the initial serum sample has bound (prior to addition of the regeneration solution)).

Modifications and variations of this invention will be apparent to those skilled in the art. The specific embodiments described herein are offered by way of example only, and the invention is not to be construed as limited thereby.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Schering Corporation

(ii) TITLE OF INVENTION: ASSAY FOR THE DETECTION OF ANTIBODIES AGAINST p53

(iii) NUMBER OF SEQUENCES: 4

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Schering-Plough Corporation
(B) STREET: 2000 Galloping Hill Road
(C) CITY: Kenilworth
(D) STATE: New Jersey
(E) COUNTRY: USA
(F) ZIP: 07033

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: Macintosh
(C) OPERATING SYSTEM: 7.5.3

(vii) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 60/028,533
(B) FILING DATE: 7 OCT 1996

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Gould, James M.
(B) REGISTRATION NUMBER: 33,702
(C) REFERENCE/DOCKET NUMBER: JB0672

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 908-298-5024
(B) TELEFAX: 908-298-5388

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
Cys Glu Pro Pro Leu Ser Gln Glu Thr Phe Ser Asp Leu Trp Lys Leu
 1               5                  10                  15

Leu Pro Glu Asn Asn Val Leu Ser Pro Leu
            20                  25
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Cys Met Asp Asp Leu Met Leu Ser Pro Asp Asp Ile Glu Gln Trp Phe
 1               5                  10                  15

Thr Glu Asp Pro Gly Pro Asp Glu Ala Pro Arg
            20                  25
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
Cys Glu Ala Leu Glu Leu Lys Asp Ala Gln Ala Gly Lys Glu Pro Gly
 1               5                  10                  15

Gly Ser Arg Ala His Ser Ser His Leu Lys
            20                  25
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Cys Ser Lys Lys Gly Gln Ser Thr Ser Arg His Lys Lys Leu Met Phe
 1               5                  10                  15

Lys Thr Glu Gly Pro
            20
```

WHAT IS CLAIMED IS:

1. A method for detecting antibodies capable of binding to p53 protein comprising:

A) immobilizing a peptide directly onto a flowcell of a sensorchip in a biosensor, said peptide capable of specifically being bound to an anti-p53 antibody,

B) obtaining a serum sample from a patient to be tested and diluting the serum sample in a suitable buffer,

C) contacting the diluted serum sample with the immobilized peptide, and

D) measuring binding of antibodies to the immobilized peptide by means of the biosensor.

2. The method of claim 1 wherein, in step A, a plurality of selected immunogenic peptides are directly immobilized, each on its own separate flowcell.

3. The method of claim 2 wherein a first peptide is selected from the amino terminal region of the p53 protein and a second peptide is selected from the carboxy terminal region of the p53 protein.

4. The method of claim 1 wherein the peptide is selected from the group consisting of:

a) a peptide comprising SEQ ID NO: 1, or a peptide having substantial sequence identity thereto;

b) a peptide comprising SEQ ID NO: 2, or a peptide having substantial sequence identity thereto;

c) a peptide comprising SEQ ID NO: 3, or a peptide having substantial sequence identity thereto; and

d) a peptide comprising SEQ ID NO: 4, or a peptide having substantial sequence identity thereto.

5. The method of claim 3 wherein the first peptide comprises SEQ ID NO: 1, and the second peptide comprises SEQ ID NO: 4.

6. The method of claim 5, further comprising a third peptide comprising SEQ ID NO: 2 and a fourth peptide comprising SEQ ID NO: 3.

7. The method of claim 1 wherein the peptide has a phosphoserine residue.

8. The method of claim 1 wherein the peptide has a phosphothreonine residue.

9. The method of claim 5, wherein the first peptide is immobilized using thiol coupling.

10. The method of claim 5, wherein the second peptide is immobilized using amine coupling.

11. The method of claim 6, wherein the first, third and fourth peptides are immobilized using thiol coupling and the second peptide is immobilized using amine coupling.

12. The method of claim 1 wherein the serum sample is simultaneously contacting with a plurality of immobilized peptides.

13. The method of claim 1 wherein the serum sample is diluted within a range of 1:2 to 1:500 in a HEPES buffered saline solution containing P-20 and carboxymethylated dextran prior to simultaneously contacting the diluted serum sample with four immobilized peptides.

14. The method of claim 2 wherein the amount of antibody that binds to each peptide is directly proportional to the response units that are reported by the biosensor.

15. The method of claim 1 wherein the immobilized peptides on the flowcells are regenerated, in preparation for analysis of a subsequent serum sample, by applying a HCl-SDS solution to remove the bound antibody.

5

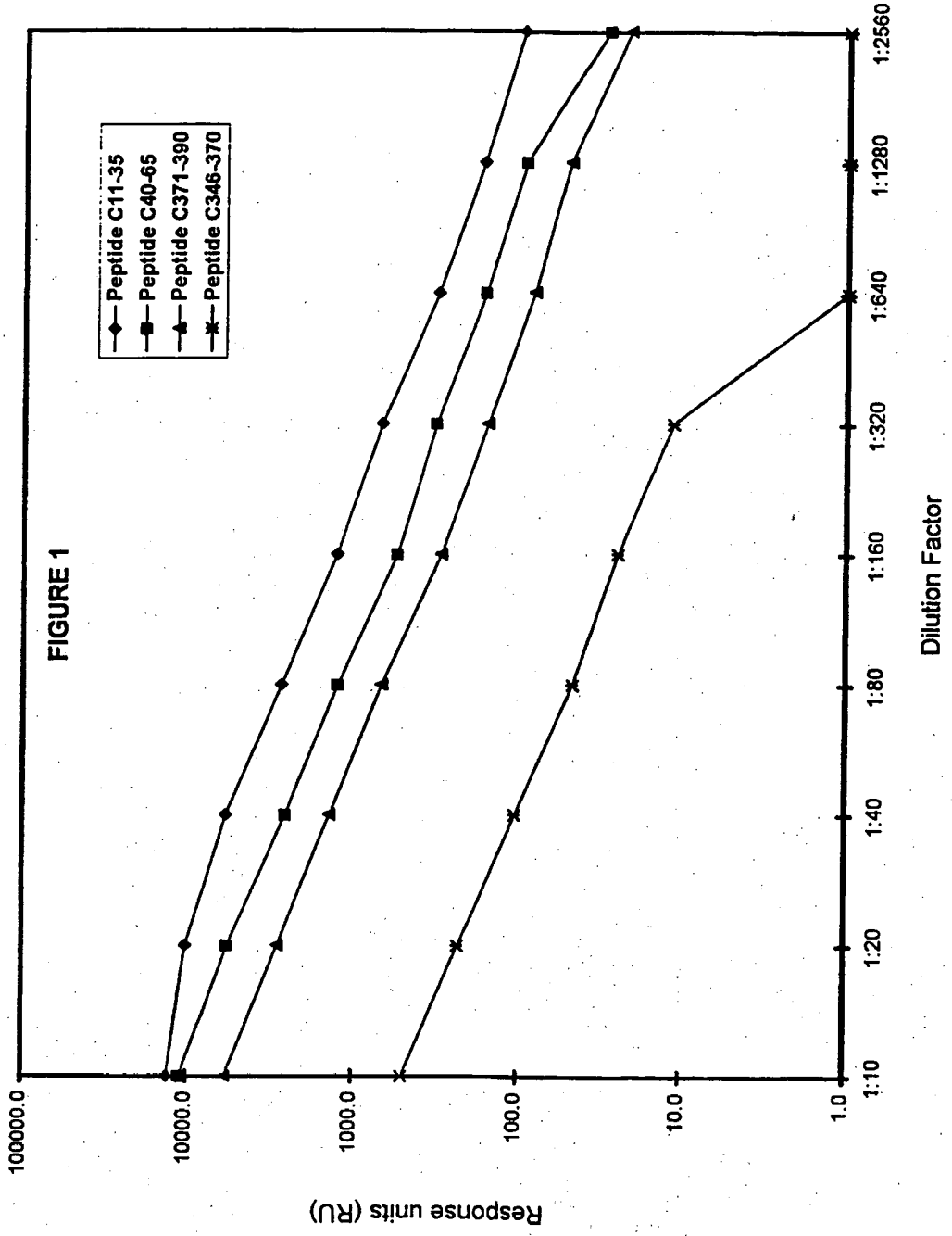
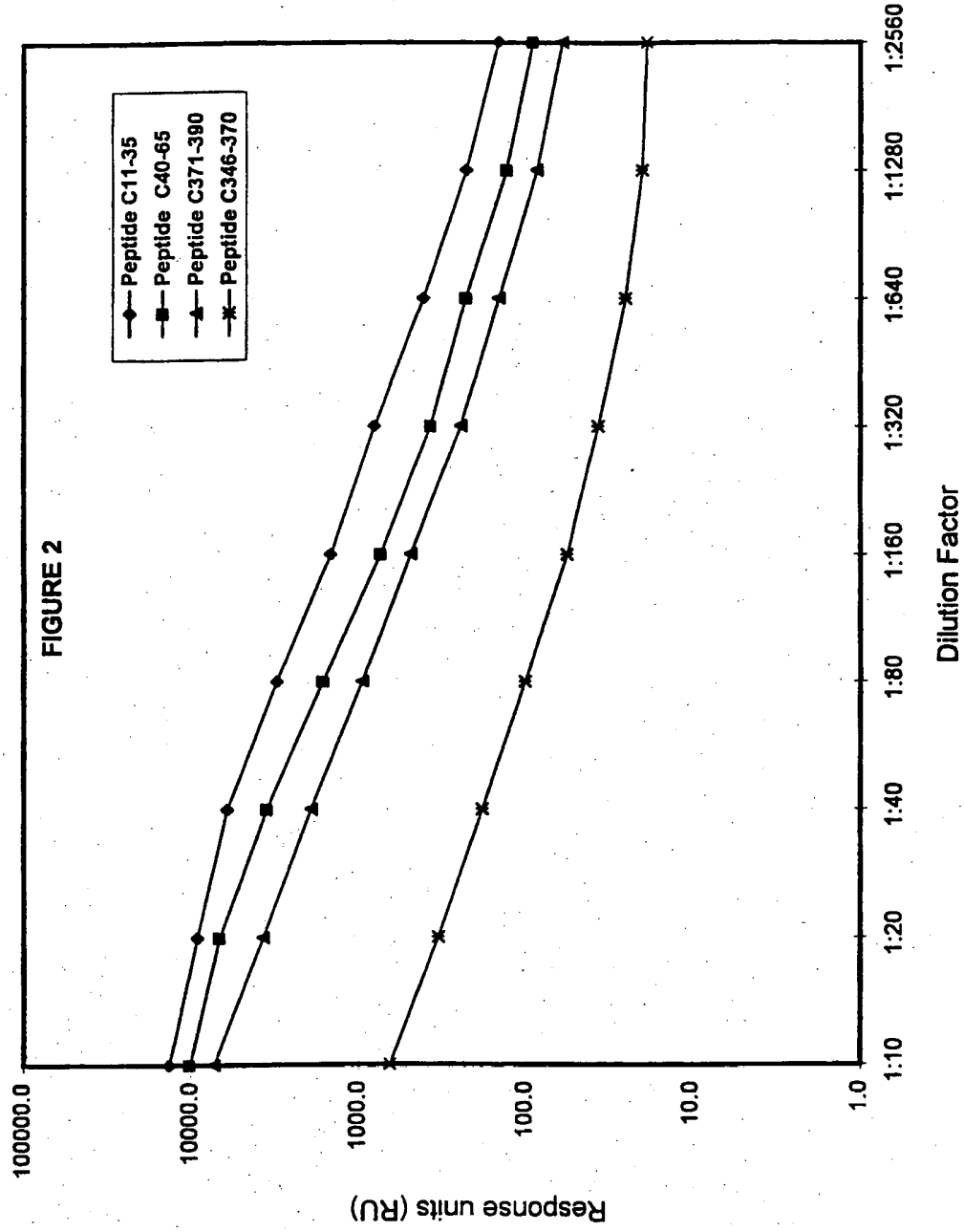

Peptide C11-35
Peptide C40-65
Peptide C371-390
Peptide C346-370
100000.0
10000.0
1000.0
100.0
10.0
1.0
Response units (RU)
1:10
1:20
1:40
1:80
1:160
1:320
1:640
1:1280
1:2560
Dilution Factor

FIGURE 1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Peptide C40-65
Peptide C371-390
Peptide C346-370
100000.0
10000.0
1000.0
100.0
10.0
1.0
Response units (RU)
1:10
1:20
1:40
1:80
1:160
1:320
1:640
1:1280
1:2560
Dilution Factor

FIGURE 2

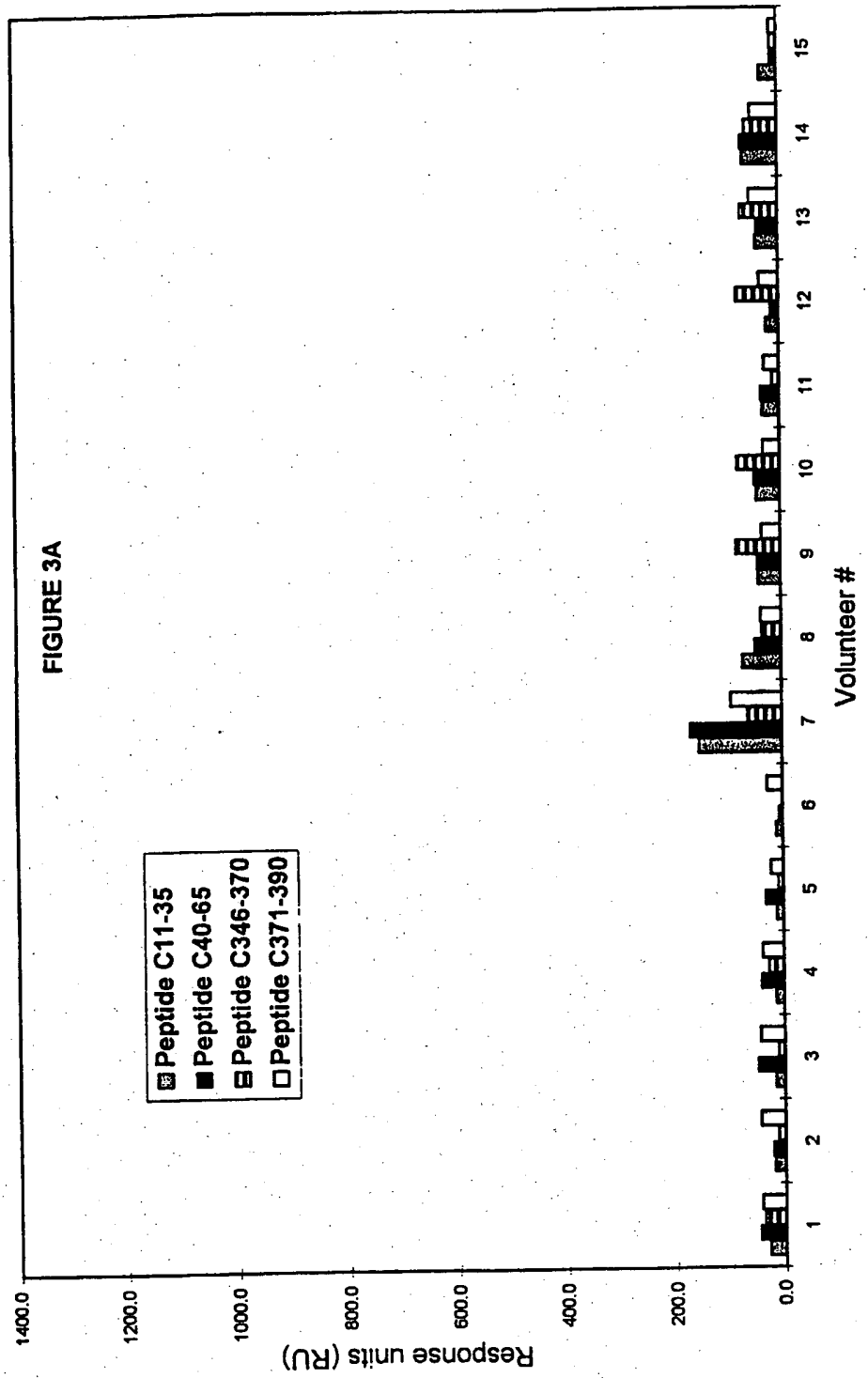
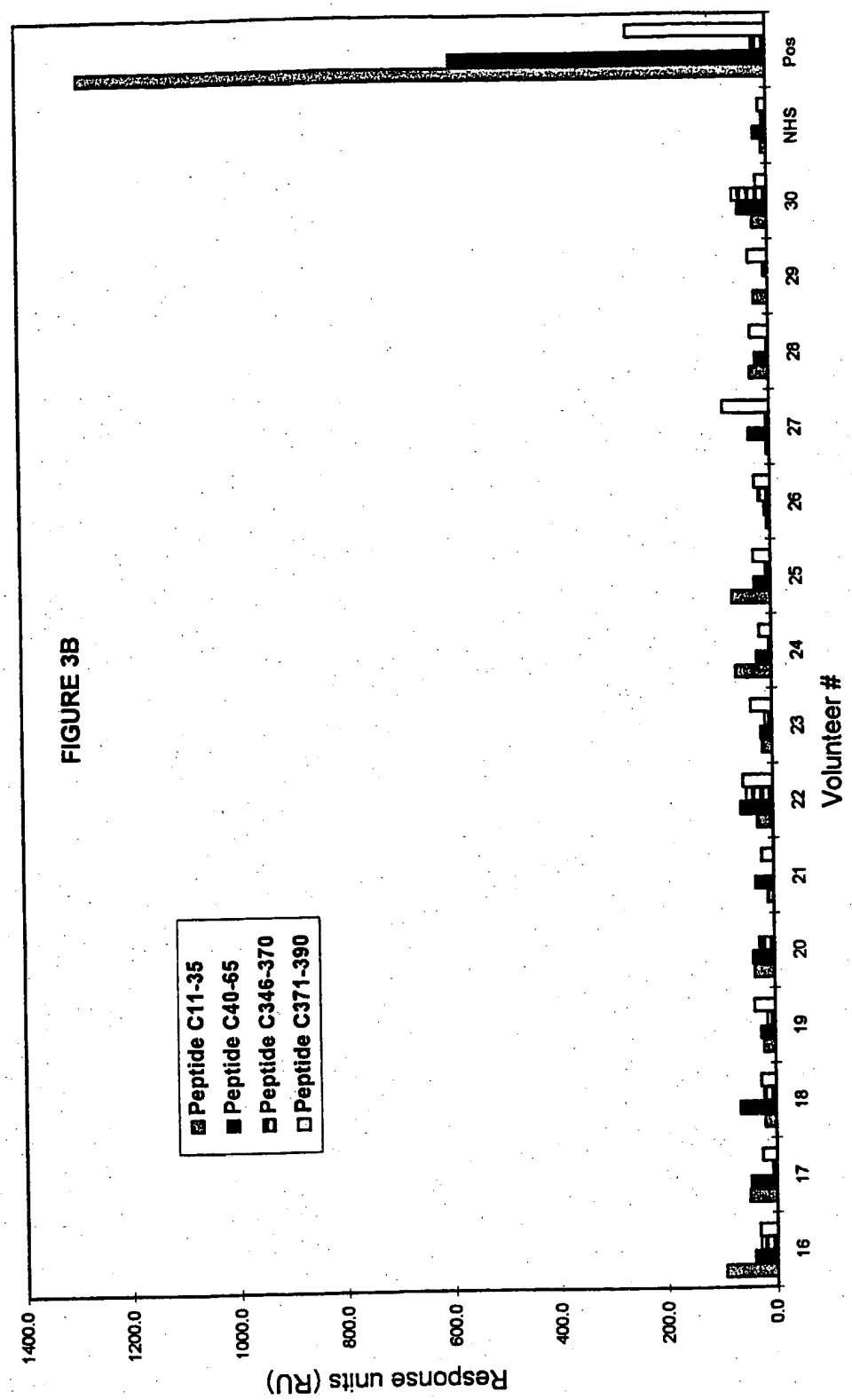

Peptide C11-35
Peptide C40-65
Peptide C346-370
Peptide C371-390
Response units (RU)
0.0
200.0
400.0
600.0
800.0
1000.0
1200.0
1400.0
Volunteer #
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15

FIGURE 3A


Response units (RU)
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1200.0
1000.0
800.0
600.0
400.0
200.0
0.0
Volunteer #
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
NHS
Pos
Peptide C11-35
Peptide C40-65
Peptide C346-370
Peptide C371-390

FIGURE 3B

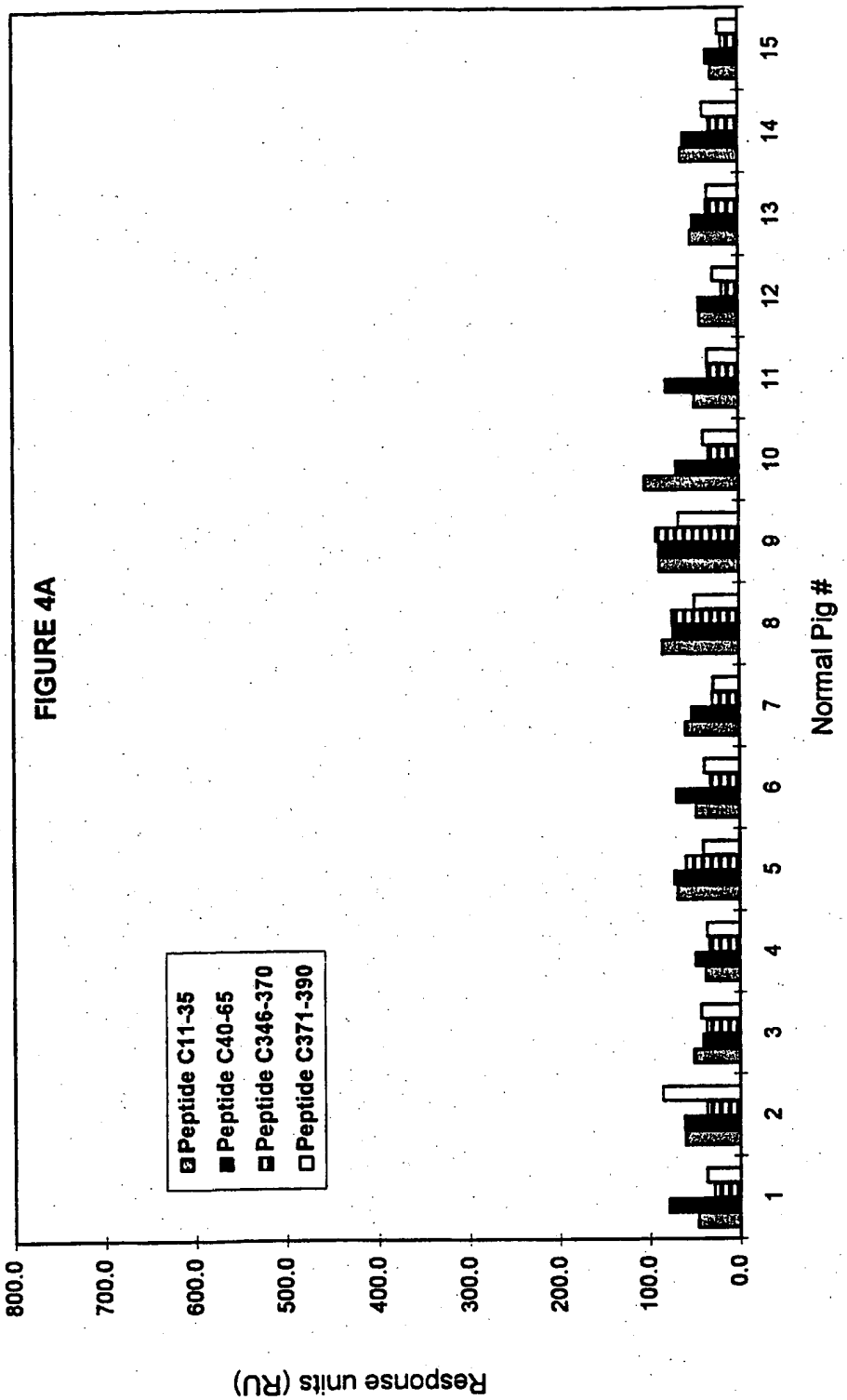
Response units (RU)
800.0
700.0
600.0
500.0
400.0
300.0
200.0
100.0
0.0
Peptide C11-35
Peptide C40-65
Peptide C346-370
Peptide C371-390
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
Normal Pig #

FIGURE 4A

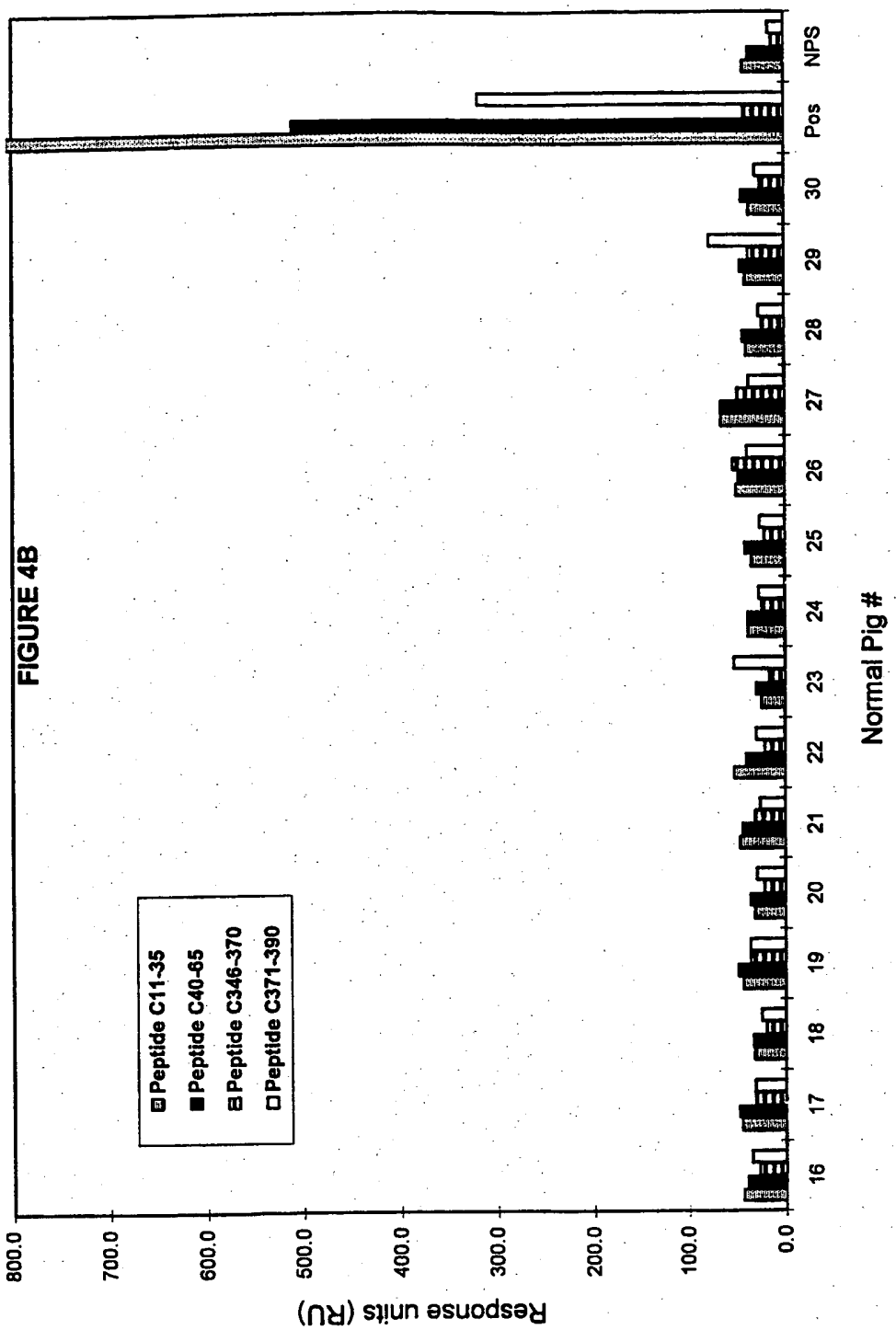
Response units (RU)
800.0
700.0
600.0
500.0
400.0
300.0
200.0
100.0
0.0
Peptide C11-35
Peptide C40-65
Peptide C346-370
Peptide C371-390
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
Pos
NPS
Normal Pig #

FIGURE 4B

INTERNATIONAL SEARCH REPORT

Int ional Application No
PCT/US 97/16132

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/574 G01N33/543

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 94 10306 A (EUROBIO LAB ;SOUSSI THIERRY (FR); LUBIN RICHARD (FR); LEGROS YANN) 11 May 1994 cited in the application see the whole document	1-15
Y	EP 0 668 502 A (YISSUM RES DEV CO) 23 August 1995 see the whole document	1-15
Y	STOCKLEY P G: "Biomolecular Interaction Analysis" TRENDS IN BIOTECHNOLOGY, vol. 14, no. 2, February 1996, page 39-41 XP004035812 see the whole document	1-15

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
20 January 1998	02/02/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hoekstra, S

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/US 97/16132

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
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